PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
A61K 31/70, 31/17, 31/495, 39/12, 38/20 // (A61K 31/70, 31:495, 31:17)

A1

(11) International Publication Number: WO 99/47146

(43) International Publication Date: 23 September 1999 (23.09.99)

(21) International Application Number: PCT/US98/05092

(22) International Filing Date: 17 March 1998 (17.03.98)

(71)(72) Applicants and Inventors: LISZIWIEWICZ, Julianna [DE/US]; 4400 East West Highway, Bethesda, MD 20814 (US). LORI, Franco [IT/US]; 4400 East West Highway, Bethesda, MD 20814 (US).

(74) Agent: LOOPER, Valerie, E.; Law Offices of Valerie E. Looper, 11726 Lightfall Court, Columbia, MD 21044 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: ANTI-HIV COMBINATION COMPRISING HYDROXYUREA, DDI, AND A PROTEASE INHIBITOR

(57) Abstract

The combination of hydroxyurea (HU), 2',3'–dideoxyinosine (ddi) and a protease inhibitor is capable of reducing the presence of the virus in both plasma and lymph nodes, as well as seminal fluids, the typical mode of transmission of the disease. An advantage of the present invention is that it can be used very early after infection to prevent seroconversion of a person infected with HIV, as well as after seroconversion. A further advantage is that the combination has relatively low toxicity, and may be suitable as a long–term treatment for chronic infection for a wide range of individuals. Yet another advantage is that, in addition to reducing the viral load in plasma and in the lymph nodes to undetectable levels, the present invention has been shown to inhibit viral rebound after treatment is stopped. An even further object of this invention is to provide a method of activating quiescent cells harboring integrated viral DNA and controlled conditions for the purpose of eliminating the integrated viral DNA.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

ANTI-HIV COMBINATION COMPRISING HYDROXYUREA, DDI, AND A PROTEASE INHIBITOR

Field of the Invention

The present invention relates generally to the field of treatment of human beings with Human Immunodeficiency Virus (HIV) infections. The inventors have found that the combination of hydroxyurea (HU), a nucleoside analog, and a protease inhibitor is capable of reducing the presence of the virus in both plasma and lymph nodes. Further, an individual has been shown to have no sign of viral rebound in plasma after discontinuing treatment for at least five weeks.

Background of the Invention

Viruses are microorganisms that depend, to some degree, on host cell components for their growth and replication. Viral infection and replication in host cells generally results in disease, whether the host is an animal or plant. Human diseases caused by viral infections include the acquired immunodeficiency syndrome (AIDS) and hepatitis. A general discussion of this field is presented in *Fundamental Virology, Second Edition*, (ed. B. N. Fields, D. M. Knipe, R. M. Chanock, M. S. Hirsh, J. L. Melnick, T. P. Monath, and B. Roizman, Raven Press, Ltd., New York, N.Y. 1991).

Retrovirus Replication

Retroviruses comprise a large family of viruses that primarily infect vertebrates. Many diseases, including the induction of some tumors, are

associated with retroviral infection (see *Fundamental Virology*, *supra*, pp. 645-708). All retroviruses, regardless of their clinical manifestations, have related structures and modes of replication.

Retroviruses contain an RNA genome that is replicated through a DNA intermediate. Inside the cell, the viral genome serves as a template for the synthesis of a double-stranded deoxyribonucleic acid (DNA) molecule that subsequently integrates into the genome of the host cell. This integration occasionally results in the induction of a tumor in the infected host organism. Following integration, a complex sequence of events leads to the production of progeny virions which are released from the infected cell.

Early in the retroviral life cycle, the RNA genome is copied into DNA by the virally encoded reverse transcriptase (RT). This enzyme can use both RNA and DNA templates, thereby producing the first strand of DNA (the negative strand) from the infecting RNA genome and a complementary second strand (the positive strand) of DNA using the first DNA strand as a template. To synthesize these DNA strands, the RT utilizes cellular substrates called deoxynucleoside triphosphates (dNTP).

Human retroviruses can be grouped into the leukemia viruses (HTLV type viruses) and the immunodeficiency viruses (HIV type viruses). HTLV infection may lead to one form of leukemia. Acquired immunodeficiency syndrome (AIDS) is caused by a form of HIV, with HIV-1 being more virulent than HIV-2. Both HTLV and HIV infect peripheral blood lymphocytes (PBL).

HIV Infection

HIV-1 was first identified as the causative agent of AIDS in 1983. The AIDS pandemic is now one of the most serious health problems worldwide. Catastrophic medical and social consequences are likely to extend into the next century. The World Health Organization (WHO) has estimated that between eight and ten million people are currently infected with HIV, and that

approximately ten times as many individuals will be affected in the next decade. The large pool of HIV carriers makes the development of effective antiviral treatments a medical priority.

The initial HIV-1 infection may occur without accompanying symptoms, but most of the patients experience an acute HIV syndrome within 2 to 6 weeks of exposure to the virus. This syndrome is characterized by fever, headaches, sore throat with pharyngitis, generalized lymphadenopathy and rashes. During this phase the virus is replicating abundantly and is detectable in the blood and the CD4+ T-cell number falls from a normal amount of 1000/mm^3 to about 500/mm^3. Antibdies to HIV-1 proteins appear in the serum between 2-12 weeks after primary infection. The sequence of appearance of these antibodies can be followed by the Western blot test, which detects the serum antibodies that bind to specific viral proteins. A positive Western blot response to gp160, gp120, p65, p55, gp41, p32, p24 and p18 proteins demonstrates that antibodies to various HIV-1 proteins are being produced. The process of change from negative for all the proteins to positive for the entire set is referred to as seroconversion. It has recently been demonstrated that during seroconversion there is a high level of virus present in the blood. The cellular arm of the immune response is also activated during seroconversion. (Borrow et al. Nature Medicine 3:(2) 212-217, 1997; Goulder et al. Nature Medicine 3:(2) 205-211, 1997). Both humoral and cellular immune response together are associated with the decline of viral load in body fluids, or viremia, during acute primary infection. In the absence of antiviral therapy, the immune system can partially control viremia. When the viremia decreases in the blood, the CD4+ T-cell number rises, but absent effective treatment, the T-cell population never fully recovers to the normal level.

Viral load, measured as HIV-1 RNA is the best available indicator of disease progression and reduced concentration of HIV-1 in various tissues

and fluids in response to antiretroviral therapy is predictive of improved prognosis (Mellors, J.W. et al. Science 272(5265) 1167-1170, 1996).

Antiviral Therapies

There is a critical need to develop effective drug treatments to combat RT-dependent viruses such as HIV. Such efforts were recently urged in the United Kingdom-Irish-French Concorde Trial conclusions which reported that the nucleoside analog zidovudine (AZT), a mainstay in the treatment of patients infected with HIV-1, failed to improve the survival or disease progression in asymptomatic patients. Other nucleoside analogs, such as 2',3'-dideoxyinosine (ddI) are currently under evaluation. The effects of ddI on disease progression and patient survival endpoints have not been adequately investigated. Non-competitive HIV-1 RT inhibitors and HIV-1 protease inhibitors have also been recently developed. These materials have different antiviral activities and pharmacokinetics properties, but they all directly target HIV-1 proteins. Despite the high efficacy of these compounds, the initial *in vitro*/*in vivo* testing has been characterized by the rapid onset of variants of HIV-1 resistant to these drugs. These drug-resistant variants, or escape mutants, retain their virulence, and appear to play a major role in the virus' ability to eventually overwhelm the human immune system. A peculiarity of HIV is that it demonstrates an extremely high rate of both reproduction and mutation. As a direct consequence, drugs which demonstrate what would in any other context be regarded as high efficacy (99.9% reduction of viral load in plasma) have not been shown to be able to eliminate the virus from an individual's system. Further, an individual may have undetectable levels of virus as measured by viral load in plasma and biopsy of lymph nodes during treatment, and yet remain infected: once treatment is stopped, the viral rate of replication increases, and the viral load rebounds. In an attempt to obtain greater accuracy, the present inventors

have used the most sensitive test methods available. Further, testing of lymph nodes is done by extracting an entire node as opposed to a biopsy sample.

Since escape mutants play such a significant role in the development of the disease, a major focus in current efforts to find a mode of treatment for AIDS is to develop strategies that feature multiple, highly effective, concurrent attacks on HIV in an effort to completely eradicate the virus from an individual's system. The only conclusive proof of effectiveness will be lack of rebound of the viral load in the individual's tissues over time.

At present, there is much interest in trying various combinations of two, three or even four drugs simultaneously. However, it has been admitted that the number of "promising" drugs is "almost astronomical". See *Antiviral Therapy for Human Immunodeficiency Virus Infections,* E. De Clercq, Clinical Microbiology Reviews, **8:2**, Am. Soc. for Microbiology (Apr. 1995).

A triple drug combination involving the use of AZT, 3TC and protease inhibitors has been suggested for the treatment of HIV-1 infection and eradication of the virus. The efficacy of this combination is thought to originate from the potency of the protease inhibitors and the mechanism of action of the AZT/3TC combination in inhibiting the rebound of resistant mutants. However, neither the protease inhibitors nor 3TC easily penetrate to certain organs such as lymph nodes and the brain, and the combination of protease inhibitor, AZT and 3TC apparently does not completely eradicate HIV-1 in macrophages or in quiescent cells, which are major reservoirs of HIV-1. Further, patients who have interrupted therapy using AZT, 3TC and protease inhibitors and then rebounded cannot be as effectively treated with the same combination because they develop resistant mutants.

Hydroxyurea has been widely used over the last three decades for the treatment of leukemia, sickle cell anemia, and has more recently been suggested for use in the treatment of HIV infections, see *Hydroxyurea as an*

Inhibitor of Human Immunodeficiency Virus-Type 1 Replication, F. Lori, et al., Science **266**:801-805 (1994); possibly in combination with a nucleoside analog such as AZT, ddI, or ddC, although it has been admitted that clinical trials using hydroxyurea alone or in combination with nucleoside analogs will be essential to assess the actual impact of use of hydroxyurea in HIV-1 impacted patients. *Hydroxyurea and AIDS: An Old Drug Finds a New Application?* F. Lori and R. Gallo, Aids Research and Human Retroviruses Vol. 11, No. 10 Mary Ann Liebert, Inc. (1995). EPO patent publication 94918016.0 filed May 17, 1994 and corresponding to USSN 08/065,814, filed may 21, 1993, which is incorporated herein as if set forth in full, describes the administration of hydroxyurea in combination with ddI, and has reported a therapeutic effect in that CD4+T-cell populations stabilized or increased in human volunteers. This result does not necessarily demonstrate that any of the individuals were cleared of the virus, because when any patient has stopped any therapy to date, an immediate rebound of viral load has occurred.

Hydroxyurea and nucleoside analogs such as ddI have potent effects on resting cells and macrophages (ref. Lori, PNAS 93 and Science 94; Goa-Wy; Agbaria R., Driscoll, J.S.,; Missuya, H.; J. Biol-Chem. 1994 Apr 29; 269(17); 12633-8; AU: Gao-W.Y.; Shirasaka, T.; Johns, D.G.; Broder, S.; Mitsuya, H.; J.Clin. Invest. 1993 May: 91(5): 2326-33) which one can speculate represents the route of initial infection during sexual, parenteral and vertical transmission, (1. SO: Science, 1993 Aug 27:261(5125); 1179-81. 2. SO: J. Clin. Invest. 1994 Nov: 94(5): 2060-7 4. SO: J. Clin. Microbiol. 1995 Feb; 33(2); 292-7, 5. S: AIDS. 1995 May; 9(5): 427-34; 6. SO: J. Exp. Med. 1996 Apr 1; 183(4): 1851-6), and this could represent an advantage of the proposed combination.

Protease inhibitors have received much attention recently in the press as being useful in combination with other drugs such as nucleoside analogs, most especially the combination of AZT and 3TC, to inhibit HIV replication

enough to yield improved quality of life for AIDS patients. It has been reported that the viral load in the plasma of such patients is greatly reduced, but not necessarily eliminated, and that whenever treatment has been stopped, the patients have experienced an increase in viral load (rebound) within a matter of 2-3 days.

The present invention is based on the discovery that a combination of hydroxyurea, a nucleoside analog, and a protease inhibitor can be used to inhibit HIV in human beings, with greatly improved results in that viral rebound may be delayed for at least three to eight weeks or more. These results indicate that the combination may be used for the treatment of HIV infection and eradication of the virus. Again, this combination takes advantage of the potency of the protease inhibitors, especially Indinavir. The HU/nucleoside analog combination has a different mechanism of action from that of the AZT/3TC combination. Further, it has been shown that the combination of HU and the nucleoside analog ddI is unable to prevent the onset of mutant viral strains conferring resistance to ddI, but the mutants are still sensitive to standard doses of ddI in the presence of HU. In addition, HU can easily penetrate to the organs such as lymph nodes and the brain, and can completely block the replication of HIV-1 in macrophages. Yet a further advantage is that viruses which are resistant to ddI and which have escaped can be inhibited by the addition of HU. Consequently, patients who have interrupted the treatment can be repeatedly treated effectively with the combination of HU, ddI and protease inhibitors.

Brief Description of the Drawings

Figs. 1-3 are the original Western Blot test results corresponding to the data in Tables 1-3, respectively.

Detailed Description of the Invention

Hydroxyurea is one of many inhibitors of ribonucleotide reductase, an enzyme known for catalyzing the reduction of ribonucleoside diphosphates to their deoxyribonucleoside counterparts for DNA synthesis. Other ribonucleotide reductase inhibitors include guanazole, 3,4-dihydroxybenzohydroxamic acid, N,3,4,5-tetrahydroxybenzimidamide HCl, 3,4-dihydroxybenzamidoxime HCl, 5-hydroxy-2-formylpyridine thiosemicarbazones, and α-(N)-heterocyclic carboxaldehyde thiosemicarbazones, 4-methyl-5-amino-1-formylisoquinoline thiosemicarbazone, N-hydroxy-N'-amino-guanidine (HAG) derivatives, 5-methyl-4-aminoisoquinoline thiosemicarbazone, diaziquone, doxorubicin, 2,3-dihydroxylbenzoyl-dipeptides and 3,4-dihydroxylbenzoyl-dipeptides, iron-complexed 2-acetylpyridine 5-[(2-chloroanilino)-thiocarbonyl]-thiocarbonohydrazone (348U87), iron-complexed 2-acetylpyridine-5-[(dimethylamino)thiocarbonyl]-thiocarbonohydrazone (A1110U), 2'-deoxy-2'-methylenecytidine 5'-diphosphate (MdCDP) and 2'-deoxy-2', 2'-difluorocytidine 5'-diphospahte (dFdCDP), 2-chloro-9-(2-deoxy-2-fluoro-ß-D-arabinofuranosyl)-adenosine (Cl-F-ara-A), diethyldithiocarbamate (DDC), 2,2'-bipyridyl-6-carbothioamide, phosphonylmethyl ethers of acyclic nucleoside analogs, [eg. diphosphates of N-(S)-(3-hydroxy-2-phosphonylmethoxypropyl and N-2-phosphonylmethoxyethyl) derivatives of purine and pyrimidine bases], nitrosourea compounds, acylclonucleoside hydroxamic acids (e.g., N-hydroxy-α-(2-hydroxyethoxy)-1(2H)-pyrimidineacetamides 1-3, and 2-acetylpyridine 4-(2-morpholinoethyl)thio-semicarbazone (A723U)).

Hydroxyurea has been widely used in cancer therapy as a broad spectrum antineoplastic drug (R. C. Donehower, *Seminars in Oncology* 19 (Suppl. 9), 11 (1992)). Hydroxyurea is readily absorbed after oral ingestion, rapidly distributed in the body fluids, including the cerebrospinal fluid, and enters cells efficiently by passive diffusion (*Id.*). Its toxic effects are less

profound and easier to control than other chemotherapeutic drugs (*Id.*).

In human chemotherapy, hydroxyurea is currently administered using two basic schedules: (a) a continuous daily oral dose of 20-40 mg per kg per day, or (b) an intermittent dose of 80 mg per kg per every third day. Either schedule could be used in the treatment of viral infections. Given the present invention, lower dosages of hydroxyurea may also be effective in treating HIV infections. Hydroxyurea is classified as a mildly toxic drug and does not cause immunodepression. Myelotoxicity is hydroxyurea's dose-limiting toxicity. However, such toxicity can be easily monitored and it is constantly and rapidly reversible after decreasing the dose or suspending the treatment (Donehower, R.C., *Semin. Oncol.* **19**:11 (1992). By monitoring simple parameters such as peripheral cell counts, hydroxyurea can be administered for years,and sometimes for decades.

A second member of the combination of the present invention is a nucleoside analog, such as the 2',3'-dideoxyinosine (ddI) used in the Examples. Nucleoside analogs are a class of compoounds known to inhibit HIV, and ddI is one of a handful of agents that have received formal approval in the United States for clinical use in the treatment of AIDS. See Clinical Microbiology Reviews, Supra, p. 200. Like zidovudine (3'-azido-2',3' - dideoxythymidine or azidothymidine [AZT], zalcitabine (2',3' - dideoxycytidine [ddC], and stavudine (2',3' -didehydro-2',3'-dideoxythimidine [D4T], ddI belongs to the class of compounds known as 2',3' - dideoxynucleoside analogs, which, with some exceptions such as 2',3'-dideoxyuridine [DDU], are known to inhibit HIV replication, but have not been reported to clear any individual of the virus.

Currently, antiviral therapy requires doses of ddI at 500 mg per day for an adult human. Similar dosages may be used in the present invention.

However, use of the combination drugs may increase the effectiveness of these nucleoside phosphate analogs so that they can be used at lower dosages or less frequently.

Of the potential protease inhibitors, compounds such as hydroxyethylamine derivatives, hydroxyethylene derivatives, (hydroxyethyl)urea derivatives, norstantine derivatives, symmetric dihydroxyethylene derivatives, and other dihydroxyethylene derivatives have been suggested, along with protease inhibitors containing the dihydroxyethylene transition state isostere and its derivatives having various novel and high-affinity ligands at the P_2 position, including 3-tetrahydrofuran and pyran urethanes, cyclic sulfolanes and tetrahydrofuranylglucines, as well as the P_3 position, including pyrazine amides. In addition, constrained "reduced amide"-type inhibitors have been constructed in which three amino acid residues of the polypeptide chain were locked into a γ- turn conformation and designated γ-turn mimetics. Other alternatives include penicillin-derived compounds, non-peptide cyclic ureas. At present, the inventors have no basis for distinguishing among the many potential protease inhibitors that may be used in combination with HU and a nucleoside analog. The protease inhibitor used in the Examples was Indinavir sulfate, available as Crixivan™ capsules from Merck & Co., Inc, West Point, PA.

Suitable human dosages for these compounds can vary widely. However, such dosages can readily be determined by those of skill in the art. For example, dosages to adult humans of from about 0.1 mg to about 1 g or even 10 g are contemplated.

The combination of compounds of the present invention may be administered by any conventional route. Administration may be oral, intravenous, intraperitoneal, intramuscular, subcutaneous, transdermal, transmucosal (e.g., by inhalation or by means of a suppository), or by any other suitable route. Administration orally in a physiologically acceptable buffered solution is preferred. The buffered solution may be used for one or

more members of the combination, while the other member or members may be administered in another form.

The particular dosage, toxicity, and mechanism for delivery of the individual of drugs of the present invention are either already known, or can be readily determined by conventional empirical techniques, as can dosages for the combination. The combination may result in the ability to use lower amounts of one or more of the constituents. This aspect of the invention may be particularly valuable with respect to the protease inhibitors, which generally are poorly soluble in water and have poor bioavailability. The present invention may address this problem in part by allowing lower dosages. The presently preferred dosage range for HU is 300-500 mg three times a day (TID), for ddI the preferred range is 100-300 mg twice a day (BID), and for Indinavir is 800 mg TID, assuming an adult weighing about 70 kg. One of ordinary skill in the art will recognize that different dosages and intervals may be appropriate. In the case of children, dosages would tend to be lower due to their smaller mass. This combination would be expected to be particularly useful for children, as the HIV infection tends to result in more brain damage in children, and this combination has good effectiveness in crossing the blood-brain barrier.

The present invention may be used before and after acute infection, before seroconversion, and after seroconversion. In particular, the data presented herein demonstrates an early treatment of the infection that may result in a profound modification of the natural evolution of the HIV-1 infection. Further, the combination might be administered prophylactically to high-risk individuals.

In addition, the present combination allows for variation in the mode of treatment over time. The protease inhibitors are known known to be most useful in certain types of activated T-cells that are actively producing virus. They are less effective in quiescent cells. The triple combination could be used only in the initial phase of therapy until the viral load is undetectable in

the plasma (less than 200 copies per milliliter) for longer than 2 months. At this point, the protease inhibitors have very likely accessed all the virus producing cells in the reservoirs they can access and have blocked active replication of the virus. Following this phase, the HU/nucleoside analog combination can be used for therapy until the virus is completely eliminated from the body. Depending on the status of the patient, the time of the treatment can be from several months to lifelong.

Another mode of treatment would be to deliberately activate certain types of quiescent cells during intensive triple combination therapy. Certain quiescent cells do not express HIV-1 proteins, and act as particularly stubborn reservoirs for the virus. In these cells, the HIV-1 DNA is integrated and both gene expression and virus production is only activated together with the activation of the cells. The cells may remain dormant for years before they spontaneously activate, and begin producing virus particles with the same ferocious reproductive rate and mutation rate as the original, acute infection. None of the presently known drugs can eliminate integrated viral DNA. This difficulty could be overcome if these cells were activated during effective combination therapy. The cells could be activated by vaccination against any of a number of diseases known to activate such cells, including, for example, HIV-1, Hepatitis B, Influenza, and Polio vaccination. HIV-1 genetic immunization is preferred, as disclosed in USSN 60/604,627, filed February 21, 1996. Such activation should preferably take place after the elimination of active virus production (that is, after the patient's viral load is undetectable for at least 2 months). Repeated activation would be helpful to ensure that all quiescent cells harboring HIV-1 DNA had been activated.

Summary of the Invention

It is an object of the present invention to provide a method of inhibiting the replication of retroviruses such as HIV-1, HIV-2, HTLV-1 and HTLV-2 in human cells. A further object of this invention is to provide a treatment for HIV infections that reduces the presence of the virus in both plasma and the lymphoid system, and which inhibits viral rebound after cessation of treatment. It is yet a further object of this invention to provide a method of treating HIV infection which is effective in the very early, as well as later, stages of infection. Yet another object of this invention is to provide a treatment for HIV which relatively less expensive and has relatively low toxicity, therefore increasing its suitability for widespread use in a large population. An even further object of this invention is to provide a method of activating quiescent cells harboring integrated viral DNA under controlled conditions for the purpose of eliminating the integrated viral DNA.

The present inventors have found that the combination of hydroxyurea (HU), 2',3'-dideoxyinosine (ddI) and a protease inhibitor is capable of reducing the presence of the virus in both plasma and lymph nodes, as well as seminal fluids, the typical mode of transmission of the disease. An advantage of the present invention is that it can be used very early after infection to prevent seroconversion of a person infected with HIV, as well as after seroconversion. A further advantage is that the combination has relatively low toxicity, and may be suitable as a long-term treatment for chronic infection for a wide range of individuals. Yet another advantage is that, in addition to reducing the viral load in plasma and in the lymph nodes to undetectable levels, the present invention has been shown to inhibit viral rebound after treatment is stopped.

These and other objects and advantages of the present invention will become apparent through the text and examples herein.

The following Examples are presented for the purpose of illustrating the practice of the present invention. They do not limit the invention, or the claims which follow.

Examples

A key step of HIV-1 infection of lymphocytes is the conversion of the viral RNA genome into double-stranded DNA by the action of HIV-1 RT. Viral DNA synthesis differs in different states of infected lymphocytes. In quiescent cells, viral DNA synthesis can be initiated as efficiently as in activated cells. However, in contrast to the activated cells, DNA synthesis in quiescent lymphocytes may terminate prematurely (J. A. Zack, et al., *Cell* **61**:213 (1990); J. A. Zack, et al., *Virology* **66**:1717 (1992)) producing no HIV-1 progeny (Zack, et al, supra; M. Stevenson, et al., *EMBO J.* **9**:1551 (1990); M. I. Bukrinsky, et al., *Science* **254**:423 (1991)). This process results in a pool of unintegrated viral DNA (Stevenson, et al., *supra*; Bukrinsky, et al., *supra*), which can remain latent in both *in vitro* infected quiescent peripheral blood lymphocytes and *in vivo* infected resting peripheral blood lymphocytes (Zack, et al., *supra*, 1990 & 1991; Stevenson, et al., *supra*; Bukrinsky, et al., *supra*). Activation of these cells can rescue HIV-1 DNA, leading to integration and production of viral progeny (*Id.*). Incomplete viral DNA has also been found associated with HIV-1 mature infectious particles, but the biological role of this DNA is unclear (F. Lori, et al., *J. Virol.* **66**:5067 (1992); D. Trono *ibid.* **66**:4893 (1992)).

Example 1 illustrates the various methods that can be used to quantitate the replication of the HIV-1. A variety of different tests with different sensitivities are currently in use, particularly since researchers have found that older screening methods with a sensitivity of < 400 copies per milliliter plasma are simply not sensitive enough to tell whether a dangerous infection continues to exist in the individual. It has also been demonstrated that

lymphoid tissues are the major reservoirs of HIV-1,(See Pantaleo, G., Graziosi, C., Demarest, J.F., Butini. L., Montroni, M., Fox, C.H., Orenstein, J.M., Kotler D.P., Fauci, A.S. *HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease.* Nature **362**(6418): 355-358 (1993) therefore, new detection methods for HIV-1 RNA and DNA have been developed and applied to the lymph tissues. Of the newer methods, the most sensitive used herein is the nested PCR assay detecting HIV-1 DNA (sensitivity: one copy of virus per sample) applied to one half of a lymph node. Another new method is the in situ hybridization detection of HIV-1 RNA, (See Fox C.H., Cottler-Fox, M. *In situ hybridization for the detection of HIV RNA in cells and tissues.* Current Protocols in Immunology (Coligan, J., Kruisbeek, A., Margulies, D., Shevack E., Strober, W. eds), Wiley, NY, 1993; and Fox C.H., Cottler-Fox, M. *In situ hybridization in HIV research.* J. Microscop. Tech. Res. **25:**78-84, 1993.) can be applied to the other half of the lymph node. A more typical sample size as reported in the current literature would be obtained via biopsy of the lymph node rather than its complete surgical removal.

Example 1

HIV Replication

Inguinal lymph nodes were surgically removed and cut in half along a longitudinal axis. One part was fixed in formalin for in situ hybridization and the other part was frozen in liquid nitrogen. The frozen tissue was homogenized and its DNA was extracted. HIV-1 DNA was amplified by a highly sensitive polymerase chain reaction (PCR assay), described in detail in Methods in Molecular Biology, Vol. 15: PCR Protocols.

PCR SK primers SK38 and SK39 are available from Perkin-Elmer, Norwalk, CT. The PCR-reaction mixture contained the following: 500 ng of genomic DNA, 0.2 mM of each primer, 100 μM of each nucleoside triphosphate, 1.5 mM $MgCl_2$, 20 mM Tris-HCl (pH 8.4), 50 mM KCl and 1 units of Taq DNA polymerase (Boheringher Manheim Corporation, Indianapolis, IN) in a final volume of 100 μl. The cycle conditions were 95 °C for 3 minutes, 50 times (94 °C for 1.30 min, 56 °C for 1.00 min and 72 °C for 1.00 min) and 72 °C for 10 minutes.

The following PCR RT primers were designed and used by the inventors:sense-primer RT-F1 (5-GGACCTACACCTGTCAACAT-3, nucleotides 127 to 146 of HXB2 pol gene) and antisense-primer RT-R8 (5-CATTTATCAGGATGGAGTTCATA-3, nucleotides 886 to 908 of HXB2 pb gene)

The PCR-reaction mixture contained the following: 500 ng of genomic DNA, 0.2 μM of each primer, 100 μM of each nucleoside triphosphate, 2 mM $MgCl_2$, 20 mM Tris-HCl (pH 8.4), 50 mM KCl and 1 units of Taq DNA polymerase (Boheringher Manheim Corporation, Indianapolis, IN) in a final volume of 100 μl. The cycle conditions were 95 °C for 3 minutes, 50 times (94 °C for 1.30 min, 56 °C for 1.30 min and 72 °C for 1.30 min) and 72 °C for 10 minutes.

Hybridization primers:

RT - F7 GGATGGAAAGGATCACCAGC

RT - R6 TACTAGGTATGGTAAATGCAGT

NESTED-PCR (THIS CAN INCREASE THE SENSITIVITY FURTHER)

Sense-primer RT-F5 (5-CAGGAATGGATGGCCCAAAAGT-3, nucleotides 233 to 254 of HXB2 pol gene)antisense-primer RT-R12 (5-TTCATAACCCATCCAAAG-3, nucleotides 874 to 891 of HXB2 pol gene).

PCR conditions were 1 μl from the first PCR reaction, 0.4 μM of each primer, 200 μM of each nucleoside triphosphate, 1.5 mM $MgCl_2$, 10 mM Tris-HCl , 50 mM KCl, and 1 unit of Taq DNA polymerase (Boheringher), in a final volume of 50 μl.

The cycle conditions were 95° C for 3 minutes, 45 times (94° C for 30 sec., 55 °C for 30 sec. and 72 °C for 30 sec.), and 72 °C for 10 minutes.

The DNA from PCR reaction was separated on an agarose gel and visualized by Ethidium Bromide staining. Polaroid pictures were taken. To increase the sensitivity at least 100 fold, the DNA was blotted to nitrocellulose paper and hybridized with a fluorecents labeled oligonucleotide according to the manufacturer protocol (ECL 3-oligolabelling and detection systems. Amersham Life Science, Little Chalfont, England).

Primer F1 was previously described by Xiping W, Ghosh S, Taylor M, Johnson V, Emini E, Deutusch P, Lifson J, Bonhoeffer S, Nowak M, Hahn B, Saag M, Shaw G. *Viral dynamics in human immunodeficiency virus type 1 infection.* Nature 1995;373:117-122; Primer F5 was described by Saag, M.S., Emini, E.A., Laskin, O.L., Douglas, J., Lapidus, W.I., Schleif, W.A., Whitley, R.J., Hildebrand, C., Byrnes, V.W., Kappes, J.C., Anderson, K., Massari, F., Shaw, G., and the L-697 working group. *A short-term clinical evaluation of L-697,661, a non-nucleoside inhibitor of HIV-1 reverse transcriptase.* L-697,661 Working Group. N. Engl. J. Med. 1993;329:1065-72.

Genomic DNA extraction from lymph nodes.

Extraction of DNA from whole tissue was done by using a DNA extraction kit available from Stratagene, La Jolla, CA, according to the manufacturer's instructions. The only modification was that the frozen lymph nodes were first ground to a powder in a porcelain mortar under liquid nitrogen, and then the powder was transferred into a Wheaton Potter-Elvehjem tissue grinder and homogenized in a lysis buffer. Incubation with protonase was done at 37 °C overnight.

Viral load quantitation by NASBA™ in semen. Quantitation of HIV-1 RNA in semen was performed by using a NASBA™ HIV-1 RNA QT kit available from Organon Teknika, Netherlands, according the the manufacturer's protocol. Briefly, 200 μl of semen were mixed with 1.8 ml of lysis buffer and frozen until use. Nucleic acids were extracted using a guanidine thiocyanate-silica based method (Boom, R., Sol, C.J.A., Salimans, M.M.M., Jansen, C.L., Wertheim-van Dillen, P.M.E., van der Noordaa, J. (1990) *A rapid and simple method for purification of nucleic acids.* J. Clin. Microbiol. **28:**495-503 and van Gemen, B., Kievets, T., Schukkink, R., van Strijp, D., Malek, L.T., Sooknanan, R., Huisman, H.G., Lens, P. (1993) *Quantitation of HIV-1 RNA in plasma using NASBA™ during HIV-1 primary infection.* J. Virol. Meth. **43:** 177-188.) Amplification of the target HIV-1 RNA by NASBA™ was performed with primers specific for the *gag* region of the HIV-1 genome (Kievits, T., van Gemen, B., van Strijp, D., Schukkink, R., Dircks, M., Adriaanse, H., Malek, L., Sooknanan, R., Lens, P (1990) *NASBA™ isothermal enzymatic in vitro nucleic acid amplification optimized for the diagnosis of HIV-1 infection.* J. Virol. Meth. **35:** 273-286, and van Gemen, B., van Beuningen, R., Nabbe, A., van Strijp, D., Jurriaans, S., Lens, P., Kievits, T. (1994) *A one-tube quantitative HIV-1 RNA NASBA nucleic acid amplification assay using electrochemiluminescent (ECL) labeled probes.* J. Virol. Meth. **49:** 157-168.

Example 2

Six individuals were treated with the combination of hydroxyurea, a nucleoside analog, and a protease inhibitor. The general course of treatment was HU, 5-8 mg/kg TID; ddI, 200 mg BID, Indinavir 800mg/TID. Four of them were treated within 4-7 weeks following primary infection and before seroconversion, that is, when the Western Blot was not completely positive.

Three patients were treated from <1 year to >5 years after seroconversion. In all the individuals the levels of plasma viremia became undetectable within 3-25 weeks after treatment. All the data is shown below in Tables 1-6, each of which is further identified by a two-letter code. Figs. 1-3 contain the corresponding Western Blot information for patients.

Three individuals began treatment within 14 to 31 days following the onset of symptoms (DFOS) of a primary HIV-1 infection and before seroconversion. In all of these individuals, the levels of plasma viremia became undetectable within 73 to 136 DFOS (with a drop of viremia between 2.7 to 3.4 logs) and remained undetectable during the course of treatment. None of these patients fully seroconverted, despite a documented exposure to HIV-1 ranging between 163 and 236 days. Their Western Blot patterns remained almost unaltered during the course of the observation. See Tables 1-3, for results of BM, FC, and SH, and Figs. 1-3 for the original and subsequent Western Blot results. Moreover, a significant, sharp increase of the CD4/CD8 ratio and CD4 count was observed in all three patients. Lymph nodes were collected from these patients at different time points to detect HIV-1 RNA by in situ hybridization. In most cases, over 40 million cells, in only one patient (FC).

The first node of FC was analyzed 8 weeks after beginning treatment, while the virus was still detectable in the plasma, and HIV-1 RNA was mainly associated to the follicular dendritic cells. At this time, the CD4 count and CD4/CD8 ratio was normal. Later, when HIV-1 was no longer detectable in the plasma, another inguinal lymph node was obtained and analyzed as before. No HIV-1 RNA was detected at that time. Again, DNA was extracted from the half of the lymph node which was frozen and tested by PCR analysis using 2 different primers and also a nested primer (sensitivity of this test is to one copy of viral DNA per sample). The PCR was positive, indicating that FC had at least one copy of HIV-1 DNA in the lymph node. See Table 1.

In patient SH, HIV-1 became undetectable in the plasma at 105 days after treatment had begun, with a decrease in viremia of 3.2 log from the baseline. The CD4 counts and CD4/CD8 ratios increased promptly after treatment began (from 0.33 to 0.95 in 33 days). In two consecutive analyses, no RNA was detected in the lymph nodes, but at least 1 copy of viral DNA was detected at 176 days from the onset of symptoms and 145 days from the start of treatment. No significant changes were observed in the Western blot profile of SH during the course of the follow-up. See Table 2.

The third patient (BM, see Table 3) was treated starting 7 weeks after the probable date of infection. Between 19 and 22 days after starting the therapy, he interrupted the treatment for three days, concomitantly with an episode of orchitis. A rebound of plasma viremia was monitored immediately after the three day suspension. Therapy was started again, and at about week 5 after initiating treatment, HIV-1 was undetectable in the plasma. At week 16, an inguinal lymph node was analyzed and 2 RNA producing cells were found out of 44 million cells screened. At week 17, treatment was again interrupted, this time due to an episode of acute hepatitis A. Despite the massive immune stimulation due to this concomitant viral infection, this individual did not show any sign of viral rebound during the following two weeks, although a positive value was found but could not be confirmed. The same week, BM again started taking the therapy. After an additional 4-5 weeks of therapy taken at irregular intervals, the patient discontinued treatment altogether. Another lymph node was obtained 18 days following final suspension of therapy, and 2 RNA producing cells were found out of a total of 44 million cells screened. No DNA could be detected in this lymph node, even after repeated nested PCR analysis. The plasma tested positive for RNA 40 days after treatment suspension at very low levels, but this positivity could not be confirmed. During all the course of the follow-up, cell

counts did not significantly change, and the Western blot profile remained practically unchanged.

Semen of patients FC, SH and BM was tested at 141, 176, and 214 days from onset of symptoms, respectively, and HIV-1 RNA was undetectable by NASBA (sensitivity <400 copies/ml). Similarly, the semen of the other patients, when tested, showed negative results.

The viral load variations in the plasma and the changes in the CD4 and CD8 absolute/relative counts of the three patients who were treated after seroconversion did not differ significantly from those who were treated before seroconversion. Patients TD and LF showed sharp increases in CD4 counts back to normal levels and their CD4/CD8 ratios now range between 1.1 and 1.4. See Tables 4 and 5. The one patient who had the longest (>5 years) infection before therapy and started with the lowest CD4 count (330), LJ, showed marked improvement also, but progress was slower. This patient became virus negative in the plasma only after 25 weeks of treatment, and the increase of the CD4/CD8 ratio was slower and less significant than in the other individuals. The CD4/CD8 ratio remains at about 0.4 at this time. See Table 6.

The patient who had been seropositive for the longest period of time, LJ, (See Table 6) had low but detectable levels of HIV-1 RNA and proteins in the lymph node after 27 weeks of treatment.

Patient TD had been seroconverted for approximately 6 months before treatment had begun. Patient TD currently shows no traces of viral DNA or RNA in the sperm, serum, or lymph nodes. This patient has had a history of hepatitis infection in March, 1996.

These data indicate that the combination of hydroxyurea, ddI and a protease inhibitor present a potent new combination that can rapidly clear the virus from plasma and lymph nodes, and inhibit viral rebound after cessation

of treatment. Further, this combination blocks HIV-1 replication in the lymphoid system and, at least in one case, shows hope for HIV-1 eradication.

In addition, these date indicate that HIV-1 infection is treatable as an emergency disease. Patients should be tested not for seroconversion, but for free virus particles in the blood if there are any symptoms or suspicion of infection, and treated immediately with the combination therapy before seroconversion takes place. The present invention will eliminate the free virus, block new infections, restore the immune system, and may eliminated virus integration in millions of cells. This method would also be economical, as treatment would be begun earlier and be of shorter duration than treatment for people with chronic infections. The present results show that the patients treated early restored the normal lymphocyte status in short periods of time (see CD4+cells and CD4/CD8 ratio). However, the patient that had been infected for 5-9 years (LJ, see Table 6) could not as rapidly restore the lymphocyte status even after 9 months of therapy, even though virus production was completely blocked. This demonstrates that the in vivo clearance rate cannot be generalized for all treatments that apparently reduce the presence of virus in plasma. Further, the same patient, unlike the patients with fresh infections, did not demonstrate restoration of the T-cell repertoire (CD4 counts and CD4/CD8 ratios) to the normal levels. This result indicates that late in the infections, T-cells do not turn over at the same rate as they did earlier, and regeneration of T-cells may be impaired. It further suggests early treatment.

The present results also indicate that a method of eliminating quiescent cells, that is, cells which have integrated viral DNA, but do not currently express the genes or produce virus. HIV-1 DNA was measured in the lymphocytes of the patients with a highly sensitive nested PCR able to detect as little as 1 copy of viral DNA. Two patients in this group had no detectable HIV-1 DNA in the lymphoid organ and 3 had detectable DNA. Other investigators have also reported (in all cases) detectable DNA in the lymphoid

organs even in the absence of virus producing cells (Markovitz, Retrovirus Conference, 1997) The present inventors are not aware of any other patients other than the two in the present study (TD, BM) who have undetectable viral DNA in the lymphoid organs. The only common feature of these patients is that both experienced hepatitis infection. BM had Hepatitis A and TD had hepatitis B. Both infections are characterized by activation of cells which can harbor HIV-1 DNA. After activation, these cells can produce viral particles which will be mainly defective in the presence of protease inhibitors. HU and ddI will work at the early phase, inhibiting reverse transcription with two different mechanisms, consequently blocking both new infection and new DNA integration.

The present results also show that early treatment of individuals (BM, SH, FC and TD, LF) infected by HIV-1 led to a profound modification of the natural evolution of HIV-1 infection.

First, HIV-1 became and remained undetectable in the plasma. The implication of this result is highly relevant. In fact, only 8% of individuals with less than 4,350 copies of RNA per milliliter of blood plasma soon after infection developed AIDS 5 years after infection, whereas 62% of those with values greater than 36,270 progressed to AIDS (Mellors, J. W. et al., Science 272(5265): 1167-1170, 1996). In the ACTG 175 study, a decrease of 1.0 log in the concentration of HIV-1 RNA from the baseline after therapy with nucleoside analogues in patients with CD4 counts between 200 and 500 per cubic millimeter was associated significantly with a 65% reduction in the risk of AIDS or death (N.Engl. J. Med. 1996 Oct 10:335(15):1091-8). All the patients analyzed here had high levels of viral replication (between 89,390 and 487,955 copies/mL) before the treatment and this load was decreased between 2.7 and 3.4 logs, that is, below 200 copies/mL.

Second, also in the lymph node compartment, which has been described as the major reservoir of the virus, only traces of HIV-1 RNA and/or DNA could be inconstantly detected. In particular, follicular dendritic cell-associated HIV-1 found in patient FC 57 days following treatment rapidly disappeared 70 days later, indicating the rapid clearance of follicular dendritic cell-associated HIV-1 following this therapy.

Third, CD4 counts increased promptly to normal levels and CD4/CD8 ratios were normalized in patients LF, TD, FC, SH, BM, whereas these values typically fail to increase to normal.

Fourth, lack of a full seroconversion in 3 of these patients treated prior to seroconversion suggests that the replication of the virus has at least been reduced to a minimum. Of particular interest is the observation that all of the above considerations hold true even after the treatment has been suspended in one of the patients. Despite a possible smoldering expression of viral RNA, DNA was repeatedly undetectable in the lymph nodes, even with a methodology able to detect a single copy of viral DNA.

Fifth, we have recently shown on an animal model (unpublished) that an early treatment with ddI and ddI with hydroxy urea, although unable to prevent the infection of pigtail macaques by a lethal dose of SIV, reduced the viral load and rescued the animals from death. This also demonstrates that early, effective treatment can completely change the course of retrovirus infection.

The combination of hydroxyurea, 2',3'-dideoxyinosine (ddI)ecxyinosine and Indinavir during the acute primary phase of infection resulted in a very potent, long lasting block of HIV-1 replication in the blood, lymph nodes and semen and in the restoration of the immune system. In one patient, the treatment was suspended without substantial viral rebound or seroconversion.

While particular embodiments of the invention have been described in detail, it will be apparent to those skilled in the art that these embodiments are exemplary rather than limiting, and the true scope of the invention is that defined by the claims that follow.

Table 1

				FC						
Infected: End July beginning of August according to Heiko										
Starts therapy: 2 Sept 1996										
3 days break in therapy: Sept 15 - 18, 1996										
THERAPY				FC LYMPH NODES after therapy						
				D/M/Y						
2.9.96	HU	400 mg TID		p24 antigen expression		HIV-RNA in situ		HIV DNA PCR		
	ddI	200 mg BID		29.10.96						
	INDINAVIR	800 mg TID		FDC	Cells	FDC	Cells			
				-	-	+	(few) +	Not detected		
				07.01.97						
D/M/Y	bDNA/PCR	PCR (RIGHT)		-	-	-	-	positive		
23.8.96	800,000									
26.8.96	1,250,000								Seminal Fluid	
2.9.96	785,000		467855 start 2-9						D/M/Y	
9.9.96	37,000		22497 stop 15-18						07.01.97	
20.9.96	21,000		13929						<400 copies/ml	
27.9.96	500		5918							
29.10.96	500		673							
18.11.96	400		<200							
23.12.96	400		<200							
6.1.97			<200							
14.01.97	500									
29.1.97			<200							
D/M/Y	Lymph.	B-Ly	T-Ly	CD4	CD4 %	CD8	CD8 %	Ratio		
26.10.96	2,543	200	2,010	1,220	48	970	38	1.26		
18.8.96	1,336	110	900	360	26	660	51	0.56		
23.8.96	1,548	80	1,020	330	21	850	55	0.39		
27.8.96	1,974	99	1,210	474	24	1,046	53	0.45		
2.9.96	3,256	130	2,563	664	21	2,064	64	0.33		
9.9.96	2,415	242	1,771	652	27	1,232	51	0.53		
20.9.96	1,546	216	1,199	660	44	526	34	1.29		
18.11.96	2,149	279	1,624	946	44	668	32	1.38		
06.01.97	1,480	252	1,139	696	47	459	31	1.52		
29.01.96	2,416	314	1,765	621	34	670	36	0.94		
estern Blot:										
Date	HIV 1+2	gp160	gp120	p65	p55	gp41/43	p32	p24	p18	
19.06.96	±	±	-	-	-	-	-	±	-	
23.06.96	±	+	±	-	-	-	-	±	±	
02.09.96	±	+	±	-	-	-	-	+	+	
09.09.96	+	+	±	-	-	-	-	±	±	
27.09.96	+	+	±	±	-	-	-	+	+	
23.12.96	+	+	±	±	±	±	±	±	±	

Table 2

					SH						
Infection: the patient said he got infected in June 29											
He went to hospital 15 July 95 with high fever,											
15 Aug 1995 (starts therapy)											
THERAPY					SH LYMPH NODES 12 wks after therapy						
27 July, 95		400 mg TID			DM/Y						
	ddI	200 mg BID			p24 antigen expression			HIV-RNA in situ		HIV DNA PCR	
	INDINAVIR	800 mg TID			16.10.95						
					FDC	Cells	FDC	Cells			
					-	-	-	-			
DM/Y		bONA	CR (RIGHT)		07.01.97					Not detected	
					-	-	-	-		positive	
19.7.83		190000									
22.7.96		182000	319168								
24.7.96		53600007	671803								
31.7.96		87310	143435								
20.8.96			3100								Seminal Fluid
23.9.96		500	918								DM/Y
25.10.96		500	266								07.01.97
28.11.96		800	<200								>400 copies/ml
12.12.96			<200								
06.01.97		600									
26.1.97			<200								
DrM/Y	Lymph.	B.Ly	T-Ly		CD4	CD4 %	CD8	CD8 %	Ratio		
16.7.96	1,578	170	1,250		520	33	870	55	0.60		
26.7.96	1,778	120	1,400		410	23	1,190	67	0.34		
2.8.96	2,040	720	1,870		560	28	1,140	56	0.52		
20.8.96	1,911	230	1,450		560	31	1,010	53	0.58		
17.9.96	2,012	307	1,849		804	40	845	42	0.95		
25.10.96	1,816	182	1,412		672	37	708	39	0.95		
12.12.96	1,742	244	1,352		645	37	627	36	1.03		
14.01.97	2,424	267	1,798		776	32	970	40	0.80		
29.01.97	2,133	296	1,527		661	31	832	39	0.79		
Western Blot:											
Date	HIV 1+2	gp160	gp120	p66	p55		gp41/43/p32		p24	p18	
16.07.96	+	+	-	-	-		-/+		-	-	
02.08.96	+	+	-	-	-		-/+		+	-	
20.09.96	+	+	-	-	-		-/+		+	-	
17.09.96	+	+	+	-	-		-/+		+	-	
04.10.96	+	+	+	-	-		-/+		+	-	
12.12.96	+	+	+	-	-		-/+		+	-	

Table 3

					BM					
Infection 10 May 96 (broken condom)										
Symptoms: 3-7 June 96										
27 June 96: starts therapy										
Testis infection 19 July 96; stop therapy; 12-19 July 1996										
Acute hepatitis A infection: stop therapy 26 Oct. 96										
Start HU + ddI 11 Nov 1996 and start HU + ddI + indinavir 30 Nov 1996										
Takes pills very irregularly										
Stops voluntarily treatment on December 20										
THERAPY					BM LYMPH NODES after therapy					
D/M/Y				D/M/Y						
27.6.96	HU	300 mg TID								
	ddI	200 mg BID								
	INDINAVIR	800 mg TID		p24 antigen expression		HIV-RNA in situ		HIV DNA PCR		
(300 HU because the weight of the patient)				FDC	Cells	FDC	Cells			
				16.10.96						
				-	-	-	(2 pos. in 44 million	Not detected		
				01.7.97						
				-	-	-	(3 pos. in 44 million	negative		
D/M/Y	BONA(PCR)	PCR (RIGHT)		FDC=follicular dendritic cells						
21.06.96	12620	80041							Seminal Fluid	
24.06.96	11620	69390							D/M/Y	
9.7.96	<500	1099	stop (12-19)						07.01.97	
19.7.96	1134	5356		rebound, 1wk a.stop, but wks still positive					<400 copies/ml	
19.8.96	<500	<200	neg	7-8 (4-6) wks a.t.						
5.9.96	<500	<200								
1.10.96	<500	<200	stop 26 Oct	10 wks a. neg						
29.10.96	<500	<200								
1.11.96		<200								
8.11.96	<400	324; <200	starts 11 Nov	2.5wks a. stop						
11.12.96	<500	<200;<200	stop 20 Dec	4 wks a. restart (BUT IRREGULARLY)						
07.01.97	<400	<200;<200		2.5wks a. stop						
16.01.97		279;<200								
27.01.97	<500	221;<200		5 wks a. stop						
7.2.97	<500	nd								
14.2.97	7007	nd								
D/M/Y	Lymph.	B-Ly	T-Ly	CD4	CD4 %	CD8	CD8 %	Ratio		
21.6.96	1,406	210	1,020	370	26	710	50	0.52		
24.6.96	1,411	230	1,020	410	29	690	49	0.59		
26.6.96	1,965	270	1,430	570	29	940	48	0.61		
19.7.96	1,643	310	1,130	490	30	620	38	0.79		
19.8.96	1,840	280	1,330	660	35	720	39	0.90		
1.10.96	1,587	238	1,152	571	36	540	34	1.06		
1.11.96	1,907	305	1,426	572	30	801	42	0.71		
11.12.96	2,304	392	1,553	991	43	968	42	1.02		
7.1.97	2,145	429	1,471	751	35	656	32	1.09		
29.1.97	1,961	412	1,302	628	32	588	30	1.07		
7.2.97	2,306	369	1,591	736	32	853	37	0.67		
14.02.97	2,232	379	1,482	670	30	781	35	0.86		
Western Blot:										
Date	HIV 1+2	gp160	gp120	p55	p55	p41/4	p32	p24	p18	
11.6.96	±	-	-	-	-	-	-	-	-	
21.6.96	±	-	-	-	-	-	-	-	-	
26.6.96	±	-	-	-	-	-	-	-	-	
19.7.96	+	+	-	-	-	-	-	±	-	
1.10.96	+	+	-	-	±	-	-	+	-	
8.11.96	+	+	±	-	-	-	-	±	±	

Table 4

TD

Infected November 1995, seroconverted Dec 1995
Naive
Hepatitis, March 1996

THERAPY		
29.07.96	HU	300 mg TID
	ddI	200 mg BID
	INDINAVIR	800 mg TID

D/M/Y	bDNA/PCR	PCR (RIGHT)
08.03.96	47,940	
13.03.96	<10,000	
25.04.96	39,290	
23.05.96	32,980	
20.06.96	30,090	
18.07.96	45,290	
22.07.96	42,000	
22.07.96	114,000	
01.08.96	48,000	
10.09.96	750	
21.10.96	<500	"neg?"
04.11.96	<500	<200
23.12.96	<500	<200
30.1.97	<400	<200

TD LYMPH NODES AFTER THERAPY

	p24 antigen expression		HIV-RNA in situ		HIV DNA PCR
	FDC	Cells	FDC	Cells	
07.01.97	-	-	-	-	negative

Seminal Fluid
D/M/Y
07.01.97
<400 copies/ml

Date D/M/Y	Lymph.	B.Ly	T-Ly	CD4	CD4 %	CD8	CD8 %	Ratio
25.04.96	1745	90	1140	490	28	1010	58	0.49
23.05.96	1934	120	1620	620	32	1120	58	0.55
20.06.96	1885	110	1550	550	29	1060	56	0.52
18.07.96	1649	120	1340	480	29	970	59	0.49
10.09.96	2253	203	1794	879	39	1104	49	0.80
23.12.96	1560	156	1213	530	34	640	41	0.83
08.01.96	2504	225	2098	1102	44	1102	44	1.00
30.01.97	2448	269	1928	881	36	979	40	0.90
14.02.97	2545	204	2138	1069	42	1044	41	1.02

Table 5

LF

Infected May 95?
Naive

THERAPY			LF LYMPH NODES AFTER THERAPY				
20.06.96	HU	300 mg TID	p24 antigen expression		HIV-RNA in situ		HIV DNA PCR
	ddI	200 mg BID	FDC	Cells	FDC	Cells	
	INDINAVIR	800 mg TID	07.01.97				
			-		-	-	positive

Nefrolitiasis

D/M/Y	bDNA/PCR	PCR (RIGHT)
05.03.96	70,200	
15.03.96	40,950	
04.04.96	14,910	
10.5.96	27,290	
07.06.96	30,820	
12.07.96	< 500	
20.08.96	< 500	
24.09.96	< 500	
10.10.96	< 500	
08.11.96	< 400	
14.12.96	< 500	
7.1.97		<200

Seminal Fluid D/M/Y	
07.01.97	
<400 copies/ml	

Date	Lymph.	B-Ly	T-Ly	CD4	CD4 %	CD8	CD8 %	Ratio
06.03.96	2090	194	2095	693	25	1358	49	0.51
22.03.96	2772	168	2193	729	26	1374	49	0.53
10.05.96	3578	250	2650	790	22	1860	52	0.42
07.06.96	2756	190	1930	630	23	1490	54	0.42
12.07.96	2584	260	1880	750	29	1190	46	0.63
26.08.96	2268	249	1701	771	34	995	44	0.77
10.10.96	2508	201	1826	853	34	953	38	0.90
04.12.96	1888	189	1385	642	34	680	36	0.94
07.01.97	2350	235	1833	940	40	870	37	1.08

Table 6

LJ

LJ Infected either 1987 or 1991 *(5-9 years a.i.)*
Naive (never treated before)

THERAPY D/M/Y		
26.3.96	HU	300 mg TID
	ddI	200 mg BID
	RITONAVIR	600 mg BID
1.7.96	HU	300 mg TID
	ddI	200 mg BID
	INDINAVIR	800 mg TID

LJ LYMPH NODES, 27 weeks after therapy

2.10.96	size: 6.5 x 12.5	
p24		2 weeks a. neg in situ
FDC	Cells	FDC Cells
+	+	- (few) +

LJ Viral load in the plasma	
D/M/y	*PCR (RIGHT)*
21.2.96	-
25.3.96	95229
1.4.96	
23.4.96	757
7.5.96	
30.5.96	836
28.6.96	393
22.7.96	347
6.8.96	
5.9.96	
19.9.96	<200
14.10.96	<200
18.11.96	
4.12.96	<200
18.12.96	
28.1.97	<200

Seminal Fluid D/M/Y
07.01.97
<400 copies/ml

Date	Lymph.	B-Ly	T-Ly	CD4	CD4 %	CD8	CD8 %	Ratio
9.2.96	1,680	176	1,382	303	19	1,053	66	0.29
22.2.96	2,178	220	1,960	374	17	1,560	71	0.24
26.3.96	1,754	190	1,490	330	19	1,190	68	0.28
1.4.96	2,317	260	2,020	420	18	1,600	69	0.26
8.5.96	1,946	230	1,640	330	17	1,380	71	0.24
30.5.96	2,346	230	1,970	400	17	1,710	73	0.23
22.7.96	1,544	220	1,310	340	22	990	64	0.34
23.8.96	1,640	200	1,390	360	22	1,020	62	0.35
14.10.96	1,830	220	1,537	439	24	1,061	58	0.41
4.12.96	1,447	130	1,270	362	25	897	62	0.40
17.01.97	1,685	185	1,445	438	26	1,078	64	0.41
28.01.97	1,260	126	1,098	290	23	794	63	0.37

WE CLAIM:

1. A method for inhibiting replication of reverse transcriptase dependent virus in animal cells, comprising the step of administering to said cells a combination of compounds selected from the group consisting of hydroxyurea, ddI, and a protease inhibitor.

2. The method of Claim 1, wherein said cells are *in vivo*.

3. The method of Claim 1, wherein said animal cells are mammalian cells.

4. The method of Clam 1, wherein the virus is a retrovirus.

5. The method of Claim 1 wherein said virus is a human retrovirus selected from the group consisting of HIV-1, HIV-2, HTLV-1 and HTLV-II and said cells are human cells.

6. The method of Claim 4 wherein said combination of compounds is administered to a human being before acute viral infection.

7. The method of Claim 4 wherein said combination of compounds is administered to a human being before seroconversion.

8. The method of Claim 4 wherein said combination of compounds is administered to a human being after serocoversion.

9. The method of Claim 4 wherein combination of compounds is administered to a human being until the viral load in plasma is less than 200 copies per milliliter, the method further comprising the step of

continuing to administer hydroxyurea and ddI, without the protease inhibitor after the viral load in plasma becomes less than 200 copies per milliliter .

10. A method for inhibiting replication of reverse transcriptase dependent virus in animal cells, comprising the steps of administering to said cells a therapy suitable for inhibiting replication of the virus, administering to said cells during said therapy at least one agent for activating quiescent cells harboring the virus.

11. The method of Claim 10 wherein the agent is a vaccine selected from the group comprising HIV-1, Hepatitis A, Hepatitis B, Influenza or Polio.

12. The method of Claim 10 wherein the agent is a genetic immunotherapy agent.

13. The method of Claim 10 wherein the agent is interleukin 2.

14. The method of Claim 10 wherein the therapy is a combination drug therapy.

15. The method of Claim 10 wherein the therapy is selected from the group comprising AZT, 3TC and a protease inhibitor, or hydroxyurea, one or more nucleoside analogs and/or a protease inhibitor.

16. The method of Claim 15, wherein the nucleoside analog is 2',3'-dideoxyinosine.

Fig. 1

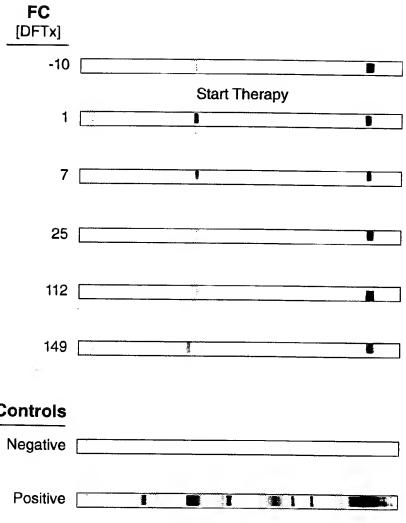

FC
[DFTx]
-10
Start Therapy
1
7
25
112
149
Controls
Negative
Positive

Fig. 2

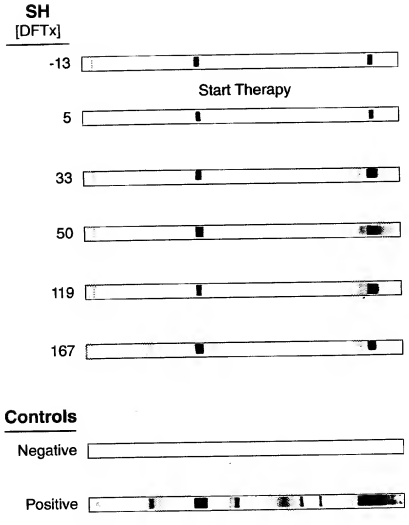
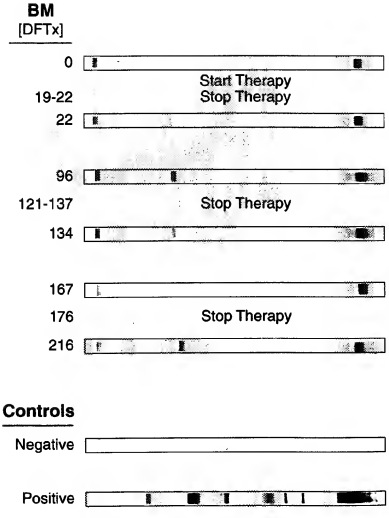

SH
[DFTx]
-13
Start Therapy
5
33
50
119
167
Controls
Negative
Positive

Fig. 3


BM
[DFTx]
0
Start Therapy
19-22
Stop Therapy
22
96
121-137
Stop Therapy
134
167
176
Stop Therapy
216
Controls
Negative
Positive

INTERNATIONAL SEARCH REPORT

Ir. .ational Application No
PCT/US 98/05092

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K31/70 A61K31/17 A61K31/495 A61K39/12 A61K38/20
//(A61K31/70,31:495,31:17)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 13314 A (UNIV LELAND STANFORD JUNIOR ;UNIV GEORGE WASHINGTON (US)) 23 June 1994 *see in particular claims 1, 9-16*	10,13-16
Y		10-16

Y	WO 94 27590 A (US HEALTH) 8 December 1994 cited in the application * see in particular examples 4,6,9; p.23, l.15 - p. 24,l. 27 *	1-16

Y	VILLA ET AL.: "Absence of viral rebound after treatment of HIV-infected patients with didanoside and hydroxycarbamide" LANCET, vol. 250, 1997, pages 635-636, XP002086710 *see the whole letter *	1-16

	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 December 1998	05/01/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Isert, B

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

I. ...ational Application No
PCT/US 98/05092

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropnate, of the relevant passages	Relevant to claim No.
Y	WO 96 23509 A (MERCK & CO INC) 8 August 1996 *see in particular claims 1,4,9-13;	1-16
Y	WO 97 31119 A (RES INST FOR GENETIC AND HUMAN) 28 August 1997 cited in the application * see in particular example 15 *	10-16
L	DATABASE AIDSLINE STN AN 1998:8140, XP002086711 (precise publication date missing) & LORI ET AL.: "Consistent, sustained HIV suppression without rebound by hydroxyurea, ddI, and a protease inhibitor prevents loss of immunologic functions" 5TH CONF. RETROVIR. OPPOR. INFECT.,1998, page 203 * abstract no. 655 *	1-9

1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Ir. ational Application No
PCT/US 98/05092

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9413314 A	23-06-1994	AU 5871694 A	04-07-1994
		CN 1094310 A	02-11-1994
		JP 7267874 A	17-10-1995
		ZA 9309217 A	03-10-1994
WO 9427590 A	08-12-1994	AU 685128 B	15-01-1998
		AU 6951994 A	20-12-1994
		CA 2163456 A	08-12-1994
		EP 0706387 A	17-04-1996
		JP 8509957 T	22-10-1996
WO 9623509 A	08-08-1996	AU 4774896 A	21-08-1996
		BR 9607714 A	13-01-1998
		CA 2211973 A	08-08-1996
		CZ 9702444 A	14-01-1998
		EP 0806957 A	19-11-1997
		FI 973184 A	30-09-1997
		HR 960045 A	31-10-1997
		HU 9800810 A	28-07-1998
		NO 973533 A	01-10-1997
		PL 321660 A	22-12-1997
		SK 103797 A	04-02-1998
WO 9731119 A	28-08-1997	AU 2136997 A	10-09-1997